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Taxines from the needles of *Taxus wallichiana*

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Abstract

A taxine, 5α*O*-(3′-dimethylamino-3′-phenylpropionyl) taxinine M (**1**) together with two known compounds 7-*O*-acetyltaxine A (**2**) and 2α-acetoxy-2′β-deacetylaustrospicatine (**3**) were isolated from the needles of the Himalayan yew, *Taxus wallichiana* Zucc. Their structures were elucidated on the basis of the NMR spectral data, ESI–MS/MS analysis and chemical methods. Compounds **1** and **3** showed moderate cytotoxic activity against the lung cancer cell line A549 in vitro.

Keywords: *Taxus wallichiana*; Taxaceae; Taxine; Cytotoxic

1. Introduction

The emergence of paclitaxel (taxol) as one of the most useful anticancer natural products has stimulated great interest in analysis of *Taxus* species with a view to finding alternative sources of this compound. As a result, numerous taxoids have been reported from various *Taxus* species (Wani et al., 1971; Chen and Kingston, 1994; Zhang et al., 2000). The needles of the Himalayan yew (*Taxus wallichiana* Zucc., Taxaceae) is one of the valuable source of taxoids (Appendino et al., 1992; Chattopadhyay et al., 1999). Recently, a new taxane IDN5109, derived from 14β-hydroxy-10-deacetylbaccatin III, is reported to have a potent antitumor activity in ovarian carcinoma (Nicoletti et al., 2000). In course of our program to isolate bio-active taxoids from the needles of *Taxus wallichiana*, we isolated a new taxine 5α-*O*-(3′-dimethylamino-3′-phenylpropionyl) taxinine M (**1**). To the best of our knowledge, this is the first benzoate containing cytotoxic taxine belonging to taxagifine type. We report herein the structure elucidation of this minor compound together with its cytotoxic activity against the lung tumor cell line A549.

2. Results and discussion

The air-dried needles (1 kg) of *T. wallichiana* were extracted with EtOH, and the EtOH extract was dissolved in EtOAc and extracted with 10% HCl. Following an acid-base partition scheme, we isolated **1** (0.5 mg) along with two other known taxines, 7-*O*-acetyltaxine A (**2**) and 2α-acetoxy-2β-deacetylaustrospicatine (**3**). Purification of these compounds was performed by semi-preparative reversed phase HPLC.

Compound **1** was obtained as a colorless solid having $[\theta]_{308}^{25}$ 2500 (0.001 M, MeOH). The HR–FAB–MS showed an intense $[M+H]^+$ ion at *m*/*z* 862.36481 which corresponded to a molecular formula of $C_{46}H_{55}NO_{15}$. Low collision energy electrospray tandem mass spectrometric (ESI–MS/MS) analysis of this compound provided a series of product ions representing diterpene ring fragments and side chains. The product ion at *m*/*z* 194.1 corresponds to a loss of 193.1 Da from the *m*/*z* 861.8 to a product ion at *m*/*z* 668.7.

Further loss of three acetic acid molecules (3×60 Da), an acetyl group (42 Da), and a benzoic acid molecule (122 Da) produced an ion at *m*/*z* 324.9. There is an another series of product ions due the loss three molecules of acetic acid, an acetyl, and a benzoic acid molecule originating from the ion at *m*/*z* 668.7. From these fragmentation patterns, it was deduced that compound **1** has at least four acetyl groups, one benzoate, and a nitrogen containing side chain (193.2 Da).

The product ion at *m*/*z* 194.1 was further characterized by the MS/MS/MS experiment. Four product ions were observed at *m*/*z* 46, 79, 107 and 149 corresponding to $(CH_3)_2NH_2^+$, $[C_6H_7]^+$, $C_7H_6OH^+$ and $C_6H_5C_2H_2COOH_2^+$, respectively. Thus these data indicated the presence of an *N*-dimethyl phenyl propionate side chain in the molecule. Furthermore, the side chain was considered to be 3-(dimethylamino)-3-phenylpropionate

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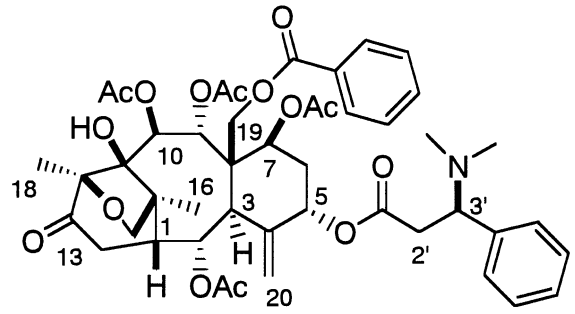
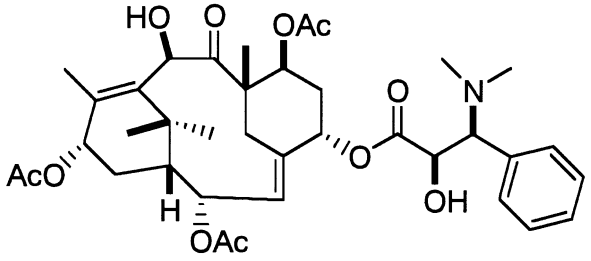
(Winterstein ester) in view of the biosynthesis of taxoid phenylisoserine side chain (Leete and Badem, 1966).



1



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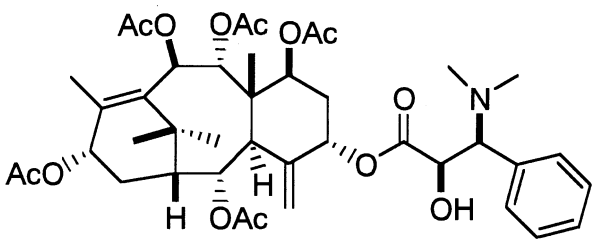

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In order to confirm the structure of this side chain, 3-dimethylamino-3-phenylpropionic acid was synthesized by reacting 3-amino-3-phenylpropionic acid with formaldehyde and sodium borohydride. The MS/MS analysis of which produced product ions identical to those of the ion 194. Thus the presence of a Winterstein ester in **1** was concluded and remaining structure elucidation was performed by its NMR spectral analysis.

A part of the ^{1}H NMR spectrum of **1** in MeOH was similar to that of taxinine M (Beutler et al., 1991), indicating the presence of a tetracyclic structure with one isolated double bond, four acetates and one benzoate group (Table 1). The connectivities of the protons in the taxane skeleton of **1** were determined by its ^{1}H–^{1}H COSY spectrum. The ^{1}H NMR spectrum of **1** showed the presence of only two methyl groups (δ 1.15 *s* and 1.26 *s*) instead of four, which is common in many taxoids, and two oxymethylenes (δ 4.10, *d*, 7.9 Hz, 3.65, *d*, 8.0 Hz, and δ 5.19, *d*, 12.2 Hz, 4.37, *d*, 12.2 Hz).

The HMQC and HMBC spectra of **1** indicated the oxygenation at C-17, with the oxygen present as an oxido bridge to C-12. The carbonyl carbon (δ 205.7) showed a long range correlation with the methyl group (δ 1.15) which showed correlations with both C-12 and C-11 in the HMBC spectrum. Thus the carbonyl carbon was assigned at C-13. The long range correlation observed in the HMBC spectrum of **1** between H-19 and the benzoate carbonyl clearly indicated the position of benzoate group at C-19. Likewise, the H_2-20 showed correlations with both C-3 and C-5, indicating an exocyclic double bond at C-4. The presence of a Winterstein ester in **1** was supported by the signals at 2.88 (3H, *s*), 2.76 (3H, *s*), 3.78 (2H, *m*), 4.95 (1H, *m*), and 7.48 (5H, *m*) in the ^{1}H NMR spectrum. Since the compound was purified

Table 1
^{1}H and ^{13}C NMR spectroscopic data of **1**

Position	δ_C[a]	δ_H
1	48.1	2.48 *m*
2	69.5	6.11 *br d*, 10.2
3	40.0	3.42 *d*, 10.2
4	140.0	
5	76.0	5.23 *br s*
6	40.0	2.0 *m*
		1.55 *m*
7	64.0	5.34 *br d*, 6.2
8	49.7	
9	70.4	5.31 *d*, 3.0
10	64.0	5.29 *d*, 3.0
11	80.2	4.15 *br s*
12	91.4	
13	205.7	
14	34.5	3.10 *dd*, 11.5, 19.2
		2.65 *d*, 19.2
15	49.7	
16	15.4	1.26
17	82.2	4.10 *d*, 7.9
		3.65 *d*, 8.0
18	11.9	1.15 *s*
19	60.8	5.19 *d*, 12.2
		4.37 *d*, 12.2
20	116.7	5.49 *s*
		4.75 *s*
$N(Me)_2$	38.8	2.76 *s*
	42.3	2.88 *s*
19-O*CO*C6H5	167.5	
19-OBz (*ipso*)	130.1	
19-OBz (*ortho*)	130.1×2	8.12 *d*, 7.9
19-OBz (*meta*)	128.6×2	7.48 *m*
19-OBz (*para*)	133.7	7.58 *m*
1′	Ob	
2′	35.9	3.78 *m*
3′	65.9	4.95 *m*
3′-Ph (*ipso*)	141.0	
3′-Ph (*ortho*)	130.1	7.48 *m*
3′-Ph (*meta*)	128.6	7.48 *m*
3′-Ph (*para*)	128.6	7.48 *m*
Ac	168.0	
	168.0×2	
	172.2	
	21.2×2	1.97 *s*, 2.11 *s*
	20.5×2	1.97 *s*, 2.06 *s*

[a] The ^{13}C NMR data were deduced from the HMQC and HMBC spectra. Direct measurement of ^{13}C NMR was not possible due to the meager quantity of the sample. δ in ppm and *J* in Hz. Ob = obscure. 1D and 2D NMR experiments (500 MHz in $CDCl_3$).

by HPLC using acetonitrile/water/TFA system, the dimethylamino group was protonated and the ^{1}H NMR signals were found to be shifted more downfield than those of similar basic taxoids.

The stereochemistry of **1** around the diterpene skeleton was deduced by comparison with taxinine M, in view the similarity in splitting pattern (coupling constants) in the ^{1}H NMR, and NOESY correlations. As in the case of taxinine M, NOESY correlations were observed between the proton pairs H-3/H-10, H-9/Me-17, H-2/Me-17, OH-11/H*b*-16, and H*a*-16/Me-17. Moreover, transannular interactions between the pairs H-3/H-10, H-9/Me-17, H-2/Me-17 may occur because the internal space may be inadequate for all the quasi-axial hydrogen atoms to fit without interacting. In this situation, some of the carbon bonds adopt eclipsed or partially eclipsed conformations (March, 1992).

The small value (ca. 3 Hz) of $J_{9,10}$ is due to an eclipsed conformation around the C-9, C-10 bond causing H-9 and H-10 to adopt an anticlinal position (Barboni et al., 1995a,b). The conformation of ring B is in the taxagifine-type owing to the presence of the C-12, C-17 oxygen bridge and the large coupling constant between H-2 and H-3 (Table 1). The signal at δ 5.23 (1H, *br s*) was characteristic of H-5α (Shi et al., 1999). The coupling constant of H-7 (δ 5.34, *br d*, 6.2 Hz) was similar to that of taxinine M. The stereochemistry at the chiral centre C-3′ was assigned as *R* from the biosynthesis of Winterstein's acid (Leete and Badem, 1966). Based on these evidences, the sterochemistry of **1** was elucidated, and the isolate was determined to be 5α- *O*-(3′-dimethylamino-3′-phenylpropionyl) taxinine M.

Compounds **2** and **3** were identified as 7-*O*-acetyltaxine A and 2α-acetoxy-2′β-deacetylaustrospicatine, respectively by comparison of their spectral data with published ones (Ettouati et al., 1988; Barboni et al., 1995a,b). Compound **1** is a minor constituent of the needles of the Himalayan yew in which taxine's concentration might be much lower than in the European or the Japanese yew (Barboni et al., 1995a,b).

We, for the first time, have attempted to elucidate the structure of a taxine through MS/MS analyses. The MS/MS analyses revealed that compound **1** is composed of four major segments, i.e. a core diterpene ring, one benzoate, four acetate, and one Winterstein ester side chain. It is interesting to note that no taxines are reported to be of the taxagifine type and compound **1** represents the first example of the taxine with a taxagifine core structure and is the second example of a taxoid involving both a benzoate and the Winterstein ester (Kobayashi et al., 1996). This type of compound possesses a different diterpene ring conformation than those of the taxol type. The unusual conformation may be responsible for diminution in biological activity compared to taxol (Chauviére et al., 1982).

Compounds **1**–**3** were evaluated for their cytotoxicity against A549 cell line and **1** and **3** showed a cytotoxicity with ED_{50} 10.1±2.4 and 28.3±3.8 μM, respectively, while **2** was inactive.

Cytotoxicity of many taxines against cancer cells has not been fully investigated. Taxinine M is reported to show a weak toxicity in the brine shrimp assay (Beutler et al., 1991). The co-occurrence of an aromatic group and an alkaloid side chain in **1** may be responsible for its cytotoxicity against A549 cells by the fact that similar activity was observed with taxuspine N (Kobayashi et al., 1996). Further elaboration on structure–activity relationships of **1** is underway.

3. Experimental

3.1. General

CD spectra were recorded with a JASCO J-710 spectropolarimeter. Mass spectrometry measurements were performed by using a Sciex API III tandem quadrupole mass spectrometer. Full-scan mass spectra were obtained while continuously scanning from *m*/*z* 300 to 1000 with a step size of 0.1 *m*/*z* and with a dwell time of 2.0 ms. MS/MS spectrum of 3-dimethylamino-3-phenylpropionic acid was obtained by selecting the protonated ion at *m*/*z* 194 at the first quadrupole (Q1), followed by collision-induced dissociation (cid) with argon gas at the second quadrupole (Q2). The resulting fragments were analyzed by the third quadrupole (Q3).

MS/MS/MS experiment of compound **1** was performed by accelerating **1** at the orifice with a high orifice potential of 150 V and by fragmenting it at Q0. The fragment with *m*/*z* 194 was selected at Q1 and was further fragmented at Q2 with argon collision gas. The fragments were analyzed by Q3. The parent scan of compound **1** in MS/MS/MS was carried out under the same condition. After fragmenting compound **1** at Q0 using orifice potential 150, the parent fragment for the ion at *m*/*z* 445 was found in the parent scan experiment. A high resolution FAB mass spectrometry was VG Micromass ZAB 2F HS. ^{1}H and 2D NMR spectra were recorded on a Bruker AMX2 500 NMR spectrometer. HPLC was performed using a Waters Delta Prep 4000 with a semi preparative RP-Vydac C-8. The eluant was monitored by the absorbance at 254 nm. Chemicals used for the synthesis of 3-dimethylamino-3-phenylpropionic acid were purchased from Aldrich.

3.2. Plant material

The needles of *T. wallichiana* were obtained from plants grown in the garden of Trichandra college, Tribhuvan University, Nepal.

3.3. Extraction and isolation

Air-dried needles (1 kg) were extracted with EtOH (4 lit ×3) at room temperature. The EtOH solvent was

evaporated under reduced pressure to obtain a semi-solid EtOH extract (110 g). A fraction of EtOH extract (50 g) was dissolved in EtOAc and extracted with 10% HCl. The acidic phase was washed with EtOAc (2 × 200 ml) and neutralized with Na_2CO_3, followed by extraction with EtOAc. After drying (Na_2SO_4), a semi-solid residue (0.5 g) was obtained which was further fractionated by the reversed-phase HPLC [a linear gradient of acetonitrile from 0 to 40% (0.6%/min) in 0.1% TFA; flow rate, 13 ml/min)]. Compounds **1**, **2** and **3** were eluted at the 40, 36 and 29% CH_3CN in the gradient system, respectively. All collected fractions were lyophilized immediately, and tested for cytotoxic activity. Those found inactive and complicated mixture were discarded. After lyophilization, 0.9 mg of **1**, 1.0 mg of **2** and 1.5 mg of **3** were obtained.

3.4. 5α-O-(3′-Dimethylamino-3′-phenylpropionyl) taxinine M (*1*)

Colorless solid, $[\theta]_{308}^{25}$ 2500 (0.001 M, MeOH). ^1H and ^{13}C NMR data, see Table 1; HR–FAB–MS *m*/*z* 862.36481 $[M+H]^+$ (required for $C_{46}H_{55}NO_{15}$ 862.36500).

3.5. Preparation of 3-dimethylamino-3-phenylpropionic acid

To a stirred solution of *dl*-3-phenyl-3-amino-phenyl propionic acid (500 mg) in H_2O/MeOH (10 ml, v/v 1:1) and formaldehyde (1 ml, 37%), $NaBH_4$ (0.7 g) was added and the mixture was stirred at 0° C for 30 min. The reaction mixture was further incubated for 5 h to give 3-dimethylamino-3-phenylpropoinic acid that was dissolved in AcOH, lyophilized and purified by reversed-phase semi-prep (yield 30%). HPLC [linear gradient (0.5%/min) of CH_3CN from 0 to 30% in 0.1% TFA with a flow rate of 13 ml/min] was used for purification. ESI–MS *m*/*z* $[M+H]^+$ 194; ^1H NMR (500 MHz, MeOH-d_4) δ 7.51 (5H, m, H-Ph), 4.79 (1H, *t*, $J=7.0$ Hz, H-3), 3.34 (1H, *dd*, $J=17.0$, 6.0 Hz, H-2a), 3.19 (1H, *dd*, $J=17.0$, 7.0 Hz, H-2b), 2.79 (6H, *s*, NMe_2).

3.6. Cytotoxic activity

A549 cells were cultivated in Dulbecco's Eagle Modified medium supplemented with 5% bovine calf serum (Hyclin). Cellular viability in the presence and absence of test samples was determined using the standard MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) method (Mosmann, 1983). Briefly, 0.2 ml cell suspension containing 2000 cells was seeded to each well of a 96-well microtitre plate (Costar, Corning Inc., NY), and incubated in a CO_2 incubator at 37 ^{0}C for 24 h before test compounds were added.

New medium with or without test compound was added to each well ($n=3$). The cells were further incubated at the same condition for 48 h. The medium was removed by aspiration, and crystals of viable cells were dissolved in DMSO (100 μl/well) and the plate was read immediately on a microtitrer plate reader (SOFTmax, Molecular Device) at the wavelength of 540 nm. Absorbance taken from cell grown in the absence of test compound was considered as a negative control.

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